Function of the Reticuloendothelial System

IV. Evidence for Two Types of Particle-Induced Reticuloendothelial Paralysis

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Reticuloendothelial system (RES) phagocytosis has been quantitated after intravenous injection of two different sets of particles by determining the clearance rate of subsequently injected identical or nonidentical particles. Injection of carbon produced a biphasic RES paralysis consisting of an early transient phase followed by a delayed sustained phase. The two phases were separated by a distinct interval of greatly augmented clearance rates. The injection of aggregated albumin was followed only by a single period of depressed clearance, which corresponded to the first phase of carbon-induced inhibition. This first phase, designated immediate RES paralysis, was initiated by particle injection and its duration was related to the rate of particle removal, to the dose of particles injected, and to the presence of the particles in the circulation. The second phase, designated delayed RES paralysis, began sometime after the particles had been engulfed by the cells, was independent of the rate of particle removal, and persisted without the presence of measurable particles in the circulation. The evidence indicates that the immediate paralysis arises from a competition between the particles in the circulation, whereas the delayed paralysis arises from a cellular derangement inhibitory to further phagocytosis. In contrast to the usual description of RES blockade as a single sustained period of depression, the present experiments indicate that the phenomenon has two phases which can be dissociated in time and mechanism.

The uptake of certain colloids by phagocytic cells in vivo can be inhibited by the prior injection of the same or a different colloid. Frequently, inert particles are used to induce this depressed state known as "reticuloendothelial (RES) blockade." Because such particles cannot be eliminated from the cell, it was thought that the decreased clearance rates represented cellular saturation and that the onset of the "blockade" dated from the progressive accumulation within the cells of the first set of particles injected (2, 3). Recently, Normann, Laguonoff, and Benditt (20) measured simultaneously the vascular clearance of two dissimilar colloids (carbon and aggregated albumin), after their sequential injection into the circulation of a rat. The clearance rate of both particles was inhibited when both were present in the circulation. Their findings could not be explained on the basis of cellular satiation and suggested that one phase of RES paralysis is associated with the immediate events arising from the presence of particles in the circulation. On the other hand, Parker and Finney (22) observed in mice that a period of normal clearance may follow particle injection and exist prior to the onset of RES paralysis. This latter observation suggests that a second period of RES depression may follow particle injection and represent some form of cellular derangement induced by colloid ingestion. Therefore, it appears probable that the usual description of "RES blockade" as a single sustained period of depression, originating with particle injection, may not be correct; rather, the phenomenon may be composed of component parts which may be dissociated in time and mechanism.

The present report examines the possibility that different phases of RES paralysis exist by investigating the behavior of the system immediately after particle exposure in contrast to the behavior evident 24 hr later. Two dissimilar colloids were studied because the response of the RES to inert colloids such as carbon may be different from the response to metabolizable colloids such as aggregated albumin. The experiments demonstrate that two periods of reduced clearance rates are associated with "RES blockade" and that the immediate paralysis initiated by particle injection is a decidedly different event.
than the delayed paralysis manifested several hours later.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing between 150 and 250 g were used after anesthesia with intraperitoneal pentobarbital sodium. The carbon suspension manufactured by Gunther-Wagner lot C-11-1431a was obtained from John Henschel and Co., New York, and used as supplied. Gelatin powder derived from swine skin was obtained from Sigma Chemical Co. (St. Louis, Mo.). Gelatin for injection was prepared as a 3% solution in 0.15 M saline. In some experiments, gelatin was added to the carbon preparation prior to its injection into animals by the method of Biozzi, Benacerraf, and Halpern (2). However, the total amount of gelatin added to carbon was held constant so that only 5 mg of gelatin per 100 g of body weight was injected into any animal irrespective of the carbon dose. Aggregated albumin was prepared by controlled heating of bovine serum albumin and was labeled with the fluorescent reagent dimethylaminonaphthelene suphonyl chloride (DNS-Cl from Sigma Chemical Co.) as described previously (20).

The vascular clearance of carbon or DNS-labeled aggregated albumin was determined by injecting intravenously a standard dose of either 10 mg of carbon or 4 mg of aggregated albumin per 100 g. After injection, blood samples were obtained from the femoral vein at regular time intervals. Clearance was measured over a 15-min period except in those instances of accelerated clearance. Blood was obtained in heparin-washed syringes with particular care taken not to introduce the anticoagulant into the circulation. The carbon concentration in each sample was determined by optical density measurement (20), where as the clearance of the aggregated albumin was measured by the decrease in plasma fluorescence (20). The plot of the logarithm of the particle concentration versus time yielded a straight line for both particle suspensions; the slope of this line was computed by linear regression and designated the clearance rate constant or K. Comparisons between the clearance rates obtained in different experiments was performed using Student's t test for comparison between group means with unequal population sizes (21).

In the studies to be described, the initial particle injection was followed by a subsequent injection of either carbon or labeled aggregated albumin. The time interval between the two injections was varied so that the effect induced by the initial injection could be evaluated at different times. Multiple determinations were made at each interval, and the value recorded represents an average determination on two or more animals.

RESULTS

Immediate RES paralysis. A typical curve representing the different rates of carbon clearance observed after injection of various doses of aggregated albumin is shown in Fig. 1. When the carbon was administered shortly after the albumin, a marked reduction in carbon clearance rate was observed for each dose. However, the rate of carbon removal was relatively constant and independent of the amount of albumin injected (K = 0.015 compared to a control of 0.036 with a standard deviation of 0.009 determined on 50 animals). A more effective paralysis of RES function as measured by carbon was not achieved by increasing the dose of aggregated albumin. On the other hand, the duration of the paralysis was dependent on the albumin dose and lengthened as the dose of the particle injected increased. For aggregated albumin doses greater than 4 mg/100 g of body weight, the paralysis terminated in a state of accelerated clearance (K greater than 0.060). Induction of the accelerated clearance state was not as effective nor was the duration sustained when the dose was reduced to 1 mg/100 g and was barely apparent at a dose of 0.5 mg/100 g. Moreover, the accelerated clearance state induced by a 10-mg dose had disappeared by 24 hr (Table 1), whereas that induced with a 30-mg dose produced significant elevation in clearance rate at that time. Thus, it appeared that the induction and the duration of the increased carbon removal rate depended upon the dose of aggregated albumin injected.

In the next series of experiments, the sequence of particle injection was reversed and the effect of an initial injection of carbon was evaluated by the clearance of a subsequent injection of labeled aggregated albumin (Fig. 2). Three different injections of carbon were made of 10, 20, and 30 mg/100 g. When the aggregated albumin was injected immediately after the carbon and essentially during its removal, the rate of albumin clearance (K = 0.004) was significantly less than the rate observed in the absence of carbon (control albumin clearance K = 0.008 with standard deviation
TABLE 1. Effect of carbon or aggregated albumin injection on the clearance rate of a subsequent injection of carbon (10 mg/100 g)

<table>
<thead>
<tr>
<th>Initial injection</th>
<th>Dose initial injection (mg/100 g)</th>
<th>Avg clearance rate K of a subsequent carbon injectiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Aggregated albumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.017 ± 0.007c</td>
<td>0.017 ± 0.005c</td>
</tr>
<tr>
<td></td>
<td>0.017 ± 0.007c</td>
<td>0.017 ± 0.007c</td>
</tr>
<tr>
<td></td>
<td>0.078 ± 0.020</td>
<td>0.078 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>0.040 ± 0.018</td>
<td>0.040 ± 0.018</td>
</tr>
<tr>
<td>Carbon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.020 ± 0.009c</td>
<td>0.020 ± 0.009c</td>
</tr>
<tr>
<td></td>
<td>0.020 ± 0.009c</td>
<td>0.020 ± 0.009c</td>
</tr>
<tr>
<td></td>
<td>0.036 ± 0.007</td>
<td>0.036 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>0.054 ± 0.020</td>
<td>0.054 ± 0.020</td>
</tr>
</tbody>
</table>

a Control: K = 0.036 ± 0.009 (50 determinations). Each value recorded represents the mean ± 1 standard deviation determined on 10 animals. Two periods of clearance depression are evident for large carbon doses, but only a single period for small carbon doses. Aggregated albumin regardless of dose produces only one period of depression.

b Hours after initial injection.

c Value significantly less than control (P < 0.01).

FIG. 2. Effect of carbon injection on the clearance rate of subsequently injected aggregated albumin. The 95% confidence limit for the normal rate of aggregated albumin removal was K = 0.0074 to K = 0.0087.

of 0.001 determined on 10 animals. Eventually, the rate of albumin clearance returned to normal and, in some instances, actually exceeded the normal clearance value. A longer period of depressed albumin clearance was observed with the larger carbon doses.

This depressant effect of carbon on RES phagocytosis was also evaluated by the clearance of a subsequent injection of the same particle. This sequence of particles was chosen because carbon is frequently used to induce "RES blockade," and the extent of the paralysis induced by carbon (and other particles) is often evaluated by a subsequent carbon injection. However, very early time intervals could not be evaluated due to residual carbon in the circulation. Accordingly, the earliest time at which the second carbon injection was made depended upon the removal rate of the first carbon dose and was given when 10% or less of the initial carbon remained in the circulation (generally 0.5 hr for 10 mg and 1 hr for 30 mg/100 g). Under these conditions, a second carbon injection was cleared at a rate slower than that observed when the same dose of carbon was given alone (Fig. 3). At 2 hr after the initial carbon injection, the rate of clearance after a 10-mg dose averaged 0.040, a value greater than the control (K = 0.036), although not significantly different.

FIG. 3. Effect of carbon injection on the clearance rate of a subsequent injection of a standardized dose of carbon. The 95% confidence limit for the normal rate of carbon removal was K = 0.033 to K = 0.039.
At the same time period, the clearance rate after an initial injection of 30 mg of carbon per 100 g was still significantly retarded ($P < 0.05$), averaging $K = 0.030$. However, the clearance rate was now recovering rapidly for, at 3 hr, greatly augmented clearance rates were observed. This elevated clearance was found after injection of 30 mg of carbon but not 10 mg of carbon per 100 g. Thus depressed clearance rates, as measured by carbon, did follow prior carbon injection, and the duration of this depression appeared to be longer with larger carbon doses.

The above experiments showed that an initial injection of either carbon or aggregated albumin delays the vascular clearance of a subsequent colloid injection, provided the second colloid is injected shortly after the first and essentially during its clearance from the circulation. This latter fact can be deduced from the observations that closely spaced injections of similar and dissimilar colloids produce inhibition, from the calculated clearances of each colloid ($dc/dt = -2.3 \, \text{Kt}$ where $c$ is concentration and $t$ is time), and from the observation that inhibition induced by carbon lasts but a short time beyond the point of detectable carbon in the circulation.

Delayed RES paralysis. Table 1 presents data comparing the immediate and delayed responses of the RES to injection of either carbon or aggregated albumin. Both a large (30 mg/100 g) and a small (10 mg/100 g) dose of each colloid were examined. Whereas the aggregated albumin produced only a single period of depressed clearance, two periods of inhibited clearance clearly existed for the larger carbon dose. The first period occurred early, coincident with particle injection, and lasted only a short time. The second period occurred late and was evident at 24 hr. The two periods of retarded clearance were clearly separated by an interval of accelerated clearance, as evident by the values reported for 6 hr. In contrast, only the first period of depression was found for the smaller carbon dose, as there was no depression of clearance rate at 24 hr; in fact, the clearance rate was actually greater than normal. Thus, a second period of clearance rate depression can occur after large doses of carbon, and the onset of the depression begins some time after the particles from the initial injection have been cleared from the circulation.

Effect of gelatin injection: a frequently used particle-stabilizing agent. Whereas aggregated albumin needs no stabilizing agent, carbon must be stabilized to prevent its flocculation in blood. Although the carbon preparation supplied by Gunther-Wagner is stabilized with partially hydrolyzed fish gelatin, many investigators—including Biozzi, Benacerraf, and Halpern (2), who developed the carbon clearance method of evaluating RES function—increase the carrier colloid presumably to render the suspension more stable in blood. It appears probable that the content of gelatin and its source might be important variables. Accordingly, gelatin alone and gelatin addition to the carbon preparation were examined for their effect on carbon removal and induction of “RES blockade.”

When gelatin was added to the carbon preparation in vitro and prior to its injection into animals, the carbon clearance rate decreased. This effect has been previously observed (10, 17). In the present experiments, the same effect could also be achieved by the injection of gelatin alone. Table 2 compares the effect upon carbon removal rate of a prior injection of gelatin alone (5 mg/100 g), of carbon alone without supplemental gelatin, and of carbon with supplemental gelatin. The following features emerged. First, the gelatin alone was sufficient to produce a prolonged depression in the clearance rate of subsequently injected carbon. This effect occurred coincident to the gelatin injection and persisted for up to 24 hr. Secondly, if gelatin were added to the carbon preparation in vitro and prior to its injection into

<table>
<thead>
<tr>
<th>Interval between 1st and 2nd injection hr</th>
<th>Avg rate of clearance of a 2nd injection of 10 mg/100 g of carbon, after a 1st injection per 100 g of*</th>
<th>5 mg of gelatin</th>
<th>30 mg of carbon</th>
<th>30 mg of carbon + 5 mg of gelatin</th>
<th>10 mg of carbon + 5 mg of gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.020 ± 0.006</td>
<td>0.030 ± 0.007</td>
<td>0.017 ± 0.005</td>
<td>0.019 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.019 ± 0.007</td>
<td>0.085 ± 0.022</td>
<td>0.014 ± 0.004</td>
<td>0.024 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.018 ± 0.006</td>
<td>0.080 ± 0.029</td>
<td>0.017 ± 0.006</td>
<td>0.022 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.023 ± 0.006</td>
<td>0.020 ± 0.008</td>
<td>0.022 ± 0.004</td>
<td>0.026 ± 0.006</td>
<td></td>
</tr>
</tbody>
</table>

* Control: $K = 0.036 ± 0.009$ (50 determinations). Each value recorded represents the mean ± 1 standard deviation determined on five animals. Gelatin confers a continuous sustained clearance depression on subsequently injected carbon.
animals, a continuous and sustained period of depressed clearance resulted irrespective of the dose of carbon injected. Thus, gelatin addition eliminated the period of normal or accelerated clearance observed between the two periods of blockade produced by an injection of 30 mg of carbon per 100 g. Further, gelatin addition to either a small or a large carbon dose produced an effect equivalent to an injection of gelatin alone (Fig. 4). Injection of the supernatant fluid of the original carbon preparation obtained after centrifugation at 45,000 X g for 2 hr had no effect upon carbon removal rate.

**DISCUSSION**

Phagocytic inhibition produced by colloid injection (RES blockade) is thought to be a single period of RES paralysis due either to a depletion of blood opsonins (8, 16, 18) or to a cellular limitation to further phagocytosis (1-3). In the present experiments, carbon injection produced a biphasic RES paralysis consisting of an early transient phase followed by a delayed sustained phase. These experiments provide evidence that RES blockade may be composed of at least two phases and each phase may involve a different limiting mechanism in phagocytosis. The phases have been designated as immediate or delayed paralysis to reflect their onset relative to the first set of particles injected. Table 3 summarizes the essential differences between the two forms of paralysis.

Whereas two phases of RES paralysis were demonstrated for carbon, the injection of a different type of particle, aggregated albumin, was followed by a single period of depressed clearance, which corresponded to the first phase of carbon induced inhibition. It would appear that the nature of the particles is an important factor in the production of the second phase of paralysis. Since delayed paralysis is initiated after particles have been cleared from the circulation, the primary effect of the particles presumably is on the cell. The ability of the cell to catabolize the particle may be an important factor because a metabolizable particle, such as aggregated albumin, would be expected to have different cellular effects than inert particles such as carbon and thorotrast or toxic particles, such as silicon dioxide (23), certain lipid emulsions (26, 27), or endotoxin (7, 14). Indeed, a prolonged sustained period of RES depression has been associated with injection of each of the latter type of particles.

Injection of either carbon or aggregated albumin was followed by an immediate period of RES paralysis. The duration of this effect was related to the rate of particle removal, to the amount of particles injected, and to the presence of the particles in the circulation. Therefore, in immediate RES paralysis, there appears to be

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**Fig. 4.** Composite graph showing the effect of carbon, gelatin, and gelatin-carbon injection on the subsequent clearance of a standard carbon dose. The different preparations were injected only once, at time zero, and the effect on the reticuloendothelial system determined in the time periods thereafter. Note that the larger carbon dose, without gelatin produces two distinct periods of clearance depression, whereas gelatin alone or gelatin with carbon produces only a single sustained period of depressed clearance.
some form of competition between the particles in the circulation, whereas, in delayed RES paralysis, there appears to be a primary cellular derangement.

The demonstration of two phases of RES paralysis depended upon producing a sufficiently short period of immediate paralysis so that a recovery period of normal or augmented clearance existed between the two phases of paralysis. Such a short period of clearance inhibition might easily be overlooked, since some particles of the first injection will be present in the circulation during this period. Adequate demonstration depends upon an observed change in the clearance rate of a second particle injected during clearance of the first. Thus, the clearance of DNS-labeled aggregated albumin was inhibited when injected during carbon clearance and, conversely, the clearance of carbon was inhibited when injected during aggregated albumin removal. Furthermore, the simultaneous determination of the clearance rates of both particles showed that there is an inhibition in the clearance rates of both particles (20). However, not all particles would possess the necessary properties to compete with each other and a certain degree of specificity in the phenomenon would be expected; indeed, such specificity has been described (13, 16, 25, 28).

Since the duration of immediate paralysis is dependent upon the numbers of particles injected as well as their rates of clearance, it should be possible to extend the period of immediate paralysis sufficiently so that an overlapping of the two periods of retarded clearance occurred. Gelatin addition to the carbon suspension prior to injection of the suspension into animals slows the rate of carbon removal. In addition, the presence of gelatin, which is very slowly removed, can itself retard the clearance of certain particles injected subsequently (13). Thus, the prolonged slow removal of carbon observed after gelatin addition would be an example of the immediate type of RES paralysis sustained by the slow removal of the first particle injected (gelatin). Because gelatin can sustain a depression in carbon clearance rate, this fact alone, or the large carbon doses used, could account for the observations of Biozzi et al. of only a single period of depression after carbon injection (2).

Recently, Jeunet and Good (9) suggested that RES depression involves both cellular and humoral factors which could be dissociated in an isolated perfused liver system. Although their conclusions are substantiated by the present study, an important distinction between the two model systems should be pointed out. Whereas repeated additions of aggregated albumin to an isolated perfused liver system produced an impaired clearance at the cellular level, repeated injections of either aggregated albumin (19) or carbon (4) in the intact animal results in faster and faster rates of clearance. That the system can actively increase its appetite for inert particles on each successive exposure in vivo indicates that, for all practical purposes, there is no limit to the capacity of the system to engulf particles. This fact renders untenable the hypothesis that the cause of delayed RES paralysis could be due to any form of cellular saturation (2), despite the historic acceptance of this theory. Therefore, a different form of cellular derangement must be involved. One possibility is a temporary failure of the cell to produce blood factors essential for phagocytosis. Pisano et al. (24) reported that puromycin pretreatment prevents recovery of clearance rates depressed by injection of a test

### Table 3. Summary of two types of particle-induced reticuloendothelial (RES) paralysis in rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Immediate RES paralysis</th>
<th>Delayed RES paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset</td>
<td>Immediate, coincident with particle injection</td>
<td>Delayed, develops sometime (hours) after particle injection</td>
</tr>
<tr>
<td></td>
<td>Metabolizable or inert Carbon, aggregated albumin, gelatin</td>
<td>Inert or toxic Carbon, thorotrast, endotoxin, certain lipid emulsions</td>
</tr>
<tr>
<td>Particle types</td>
<td>Usually short (minutes to hours) but may be prolonged; duration dependent upon particle dose, rate of removal, and presence of particles in circulation</td>
<td>Prolonged (hours to days); duration not dependent upon dose or presence of circulating particles</td>
</tr>
<tr>
<td>Examples of particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>Two-particle interaction in circulation as (i) adsorption onto particle surface, or (ii) competition for phagocytic sites or opsonin</td>
<td>Unknown Cellular derangement as (i) temporary failure to synthesize opsonin or replace cell constituents, or (ii) sustained failure requiring cell replacement for recovery</td>
</tr>
<tr>
<td>Particle specificity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible mechanism</td>
<td></td>
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</tr>
</tbody>
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lipid emulsion and concluded that new synthesis of opsonic protein may be involved in recovery. Alternatively, there could be a need for synthesis of new cell membrane or other cellular constituents consumed in the process of phagocytosis. Finally with more extensive cellular derangement, there may need to be a replacement of cells to effect recovery. Kelly and co-workers (11, 12) showed that ingestion of particles by cells results in a marked increase in incorporation of tritiated thymidine into deoxyribonucleic acid of Kupffer cells, and it has been reported that nitrogen mustard, by blocking cell replication, can prolong RES blockade (1).

The immediate RES paralysis that occurs when two particles are in the circulation involves some form of interaction between the particles. However, it remains unclear how the presence of the particles actually interferes with phagocytosis, although at least three possibilities suggest themselves. (i) There is a direct interaction between the particles, with one particle being adsorbed onto the surface of the other, changing the surface properties of either or both; (ii) there is a competition between the particles at the phagocytic site on the cell (6); or (iii) there is a competition for, with resulting depletion of, available serum factors essential to optimal phagocytosis (5, 15, 18).

ACKNOWLEDGMENTS

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LITERATURE CITED


